



Therapeutic Intervention for Caseous Lymphadenitis Using Intra-abscess Instillation of Ozone or Hydrogen Peroxide in Small Ruminants

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ABSTRACT

CLA is an economically and zoonotically important disease in the world. The lack of a therapeutic procedure limits the treatment mainly to surgical intervention. Therapeutic efficacies of the intra-abscess instillation of O₃-oil and H₂O₂-gly in CLA in small ruminants were tested. One hundred eighty affected sheep and goats were allocated to five groups as follows: 1) NC (no intervention), 2) PC1 (injection of olive oil), 3) PC2 (injection of glycerin), 4) injection of O₃-oil, 5) injection of H₂O₂-gly. Samples of abscess contents were collected for microbiological examination prior to injection. The VAs were measured on T0, then with two-week intervals on T1 and T2. On T0 and T2, VAs were as NC (2.9 ± 0.5; 3.5 ± 0.5), PC1 (3.4 ± 0.7; 6.6 ± 1), PC2 (3.1 ± 0.7; 3.3 ± 0.9), O₃-oil (3.3 ± 0.4; 0.4 ± 0.4), and H₂O₂-gly (4.6 ± 0.4, 1.5 ± 0.4). Statistical analysis showed a significant decrease in VAs, merely in treatment groups. CP was recovered in 48.3% of bacteriological samples. The results of this study suggested that O₃-oil and H₂O₂-gly would be reliable therapeutic agents for treating and controlling CLA. Ozone showed apparently a higher efficacy and caused more rapid shrinkage/recession of the abscesses, compared to hydrogen peroxide..

Keywords

Abscess, Caseous lymphadenitis, Ozone, Hydrogen peroxide, Small ruminants

Number of Figures: 0
Number of Tables: 6
Number of References: 60
Number of Pages: 11

Abbreviations

CLA: Caseous lymphadenitis
CP: *Corynebacterium pseudotuberculosis*
H₂O₂: Hydrogen peroxide
O₃: Ozone

NC: Negative control
PC1: Positive control 1
PC2: Positive control 2
O₃-oil: Ozonated olive oil

Introduction

The CLA of sheep and goats caused by CP is an important problem worldwide. The chronic and insidious nature of the infection makes the control of the disease difficult, leading to a high prevalence in many parts of the world. Moreover, it causes significant economic losses in small ruminant populations [1]. The pyogenic superficial abscesses due to CLA, especially in the head and neck areas of sheep and goats, should always be considered as a source of serious concern for human health, herd health management programs, and profitability [1]. Based on the report of the United States Department of Agriculture in 2012, the national wool, mohair, and milk production economic losses were valued at \$9.39 million. Losses in milk, meat, and wool production caused by CLA may be associated with substantial economic impacts on both low- and high-income countries [2].

CLA along with four other economically important diseases of sheep and goats, namely ovine Johne's disease, Maedi-Visna, ovine pulmonary adenomatosis, and border disease, collectively have been classified as "iceberg diseases" [3]. The presence of animals with the subclinical form of infection (the most challenging type for both practitioners and owners) is a common feature of these diseases [4]. Generally, CLA is a chronic (multi)systemic infection caused by a pyogranulomatous response of the lymphoid system, which ultimately results in the enlargement and abscessation of superficial and sometimes internal lymph nodes [3, 5].

The leading cause of CLA, CP, inhabits the environment and has rarely been isolated from the skin, nasal orifices, and ear canals of apparently unaffected sheep [7,8]. The most documented route for infection is thought to be skin wounds formed during various daily procedures, including unsafe or contaminated needle puncture [9].

Ruptured superficial abscesses play a crucial role in the spread of infection, whereas for pulmonary abscesses, such a role has not yet been fully established [6]. The isolation and identification of causative micro-organism(s) is the gold standard for diagnosis. Purulent materials and exudates are the most common specimens used for diagnostic purposes [3].

Abbreviations-Cont'd

H₂O₂ gly: H₂O₂-glycerol
T0: Baseline before treatment
T1: Time 1
T2: Time 2
Vas: Volumes of abscesses
BCS: Body condition score
LSM: Least square mean
SEM: Standard error of the mean
FDA: U.S. Food and Drug Administration

Classically, the standard surgical approach, including clinical lancing to drain purulent materials, is common when the abscesses mature. In recent years, some new therapeutic alternatives, such as antimicrobial photodynamic therapy, H₂O₂, and biogenic nanoparticles have been attempted with some promising results in cancer tumors and semi-spherical masses in human medicine. However, the need for relatively sophisticated equipment prevents them from being applicable in the current large animal field settings [10-12]. It has been suggested that potent bio-oxidants, such as H₂O₂ and O₃, in biologically safe forms and doses would be suitable candidates for investigating potential therapeutic intervention effect(s) in resolving encapsulated abscesses. The unique physicochemical properties of these compounds render them an eureka with the potential to be applicable in various biomedical fields [13].

Trioxigen, or O₃ can be administered as a pure gas in aqueous solutions (ozonated saline) or ozonated vegetable oils during ozone therapy. Ozone has been administered successfully through parenteral (IV, IM, SC, and intralesional) and loco-regional (cutaneous, rectal, vaginal, nasal, and dental) or even whole-body routes in human and animal patients [14]. Bio-oxidative therapy as a viable therapeutic modality offers medical and veterinary practitioners the best opportunity to safely deal with numerous pathological conditions, which are less responsive to conventional treatments and/or often fail after prolonged, frustrating, and costly interventions [13, 15].

Considering the therapeutic potentials of O₃ and H₂O₂, it seems such agents would be active in situations, where antimicrobials fail to penetrate or remain active in purulent environments. These capabilities could enable them to potentially sterilize the internal environment of the abscesses. These materials might be good candidates to be investigated as new approaches to deal with CLA, targeting the destruction of the pyogenic membranes of the abscesses.

From a practical standpoint, O₃ is an unstable gas that cannot be stored and should be used at once because it has a half-life of 40 min at 20°C and 140 min at 0°C [14]. However, the oxidant activity of O₃ in ozonated vegetable oils will be extended up to one year at 25°C or two years in the refrigerator [16]. Hydrogen peroxide is commercially available as a topical antiseptic agent for disinfecting the skin and superficial lesions [17]. It has also been frequently used for its well-known properties for wound healing and radiosensitization in the management of soft tissue tumors [18]. Direct application of pure H₂O₂ to wounds and body cavities may potentially cause intra-arterial oxygen embolism [17] and local mild to severe pain at injection sites [19]. Sodium hyaluronate has been

used as a solvent to alleviate the potential side effects of H₂O₂ injections inside the tumors prior to radiotherapy [17,19, 20], as well as inactivating the anti-oxidative enzyme peroxidase in tumors [21]. Glycerin or glycerol have been used as solvents as well to prolong the release of H₂O₂ [22].

Attempts to expedite the maturation and subsequent drainage of the abscesses could be the hallmark to stop the further spreading of the disease. The internal abscesses may occur on some occasions, with no overt clinical signs, serving as a source of disease proliferation through the visceral form of CLA believed to be associated with clinical manifestations of the so-called "thin ewe syndrome".

Apart from the surgical intervention, as far as the authors know, few pure therapeutic interventions have been devoted to the treatment of the disease. A closed-system lavage of abscesses and the intralesional administration of tulathromycin have been suggested [23]. A combination of intramuscular injection of rifamycin and tetracycline has been reported to be effective in the resolution of the enlarged and ruptured peripheral lymph nodes discharging thick green pus [24].

Based on the current knowledge, we hypothe-

sized that the direct intra-abscess administration of either O₃ or H₂O₂ might have a beneficial effect on the treatment of CLA. The hypothetical basis is the assumption that the intra-abscess injection of a chemical agent that preserves its activity in purulent contents might have a recessive effect by sterilizing the pyogenic membrane. The objective of this field study was to study the impact of injecting either ozonated olive oil or 0.5% w/v H₂O₂ into intact CLA abscesses compared to control groups.

Result

A total of 208 affected abscesses from 180 animals [156 sheep (86.7%) and 24 goats (13.3%)] were included in this study. The distribution by treatment and the mean volumes of instilled agents are given in Table 1.

The isolated bacterial cultures from 180 animals (208 abscesses) are presented separately according to the type of isolated bacteria in each experimental group in Table 2.

The affected lymph nodes are described separately based on the type of anatomical position in each experimental group in Table 3.

The volume of the abscesses at different time intervals (T0, T1, and T2) are given in Table 4.

Volumes of abscesses differed significantly between treatments at T0 with the largest abscesses in the H₂O₂-gly group. Statistically significant

Table 1. Abscesses, animals and average volumes of instilled agents in mL by treatment

	NC	PC 1	PC 2	O ₃ -oil	H ₂ O ₂ -gly
Abscesses (animals)	28 (30)	19 (17)	17 (13)	74 (61)	70 (59)
Volumes instilled	-	2.79 ± 0.9	2.72 ± 2.1	4.36 ± 3.7	5± 4.6

Table 2. Isolated bacteria in the abscesses in all experimental groups (208 abscesses)

groups	C. pseudotuberculosis*	Streptococcus sp	(Arcanobacterium) sp	Trueperella	Staphylococcus aureus	Staphylococcus sp	Rhodococcus sp	Micrococcus sp	Actinobacillus sp	Bacillus sp	Yersinia pseudotuberculosis	Actinomyces sp	Dermatophilus congolensis	Nocardia sp	No Growth	Growth of several colony forming units were observed	total
PC1	11	1	1	1	2	1	0	0	1	0	0	0	0	0	3	2	23
PC2	10	2	0	2	0	0	1	0	3	0	0	0	0	0	1	1	20
O ₃ -oil	23	13	12	7	6	6	3	1	0	1	1	1	1	0	0	0	74
H ₂ O ₂ -gly	43	6	5	3	2	3	2	3	0	1	1	0	1	0	0	0	70
Total	87	22	18	13	10	10	6	4	4	2	2	1	1	1	3	4	
Total (%)	48.3	12.2	10.0	7.2	5.5	5.5	3.3	2.2	2.2	1.1	1.1	0.55	0.55	1.4			

* Corynebacterium pseudotuberculosis

Table 3.
Lymph node disruption involved with caseous lymphadenitis

groups	R*, mandibular	L^, mandibular	R, parotid	L, parotid	R, Retropharyngeal	L, Retropharyngeal	R, prescapular	L, prescapular	R, supra mammary (scrota)	L, supra mammary (scrota)	R, prefemoral (subiliac)	L, prefemoral (subiliac)	R, popliteal	L, popliteal	total
NC	8	3	5	3	3	1	1	2	1	0	1	0	0	0	28
PC1	5	8	0	6	0	0	0	0	0	0	0	0	0	0	19
PC2	2	6	3	4	0	0	0	2	0	0	0	0	0	0	17
O ₃ -oil	13	13	20	12	7	4	0	2	1	1	0	0	1	0	74
H ₂ O ₂ -gly	21	13	9	9	9	6	1	0	1	0	0	1	0	0	70
Total	49	43	37	34	19	11	2	6	3	1	1	1	1	0	
Total (%)	23.6	20.7	17.8	16.3	9.1	5.3	1.0	2.8	1.4	0.5		0.5	0.5	0	

* Right, ^ Left

Table 4.
The volume of the abscesses at different times are presented as LSM ± SE (mm³)

	T0	T1	T2	Within group differences
NC	2.9 ± 0.5 €	3.6 ± 0.5 ¥	3.5 ± 0.5 Σμ	= 0.366
PC1	a 3.4 ± 0.7 ¥€	b 5.6 ± 0.7 €®	b 6.6 ± 1 ®	= 0.031
PC2	a 3.1 ± 0.7 €	a 4.3 ± 0.7 ¥®	a 3.3 ± 0.9 £μ	= 0.211
O ₃ -oil	a 3.3 ± 0.4 €	b 1.9 ± 0.4 £	c 0.4 ± 0.4 €	= 0.01
H ₂ O ₂ -gly	a 4.6 ± 0.4 ¥	a 3.7 ± 0.4 ¥	b 1.5 ± 0.4 ¥	≤ 0.0001
Between group significant differences	= 0.0387	= 0.0137	= 0.0176	

Different alphabetical letters within a line represent statistically significant differences between time points. (*p* < 0.05)
Different symbols within a column represent statistically significant differences between treatments for each sampling time (*p* < 0.05)

Table 5.
Test of fixed effects. The mean volume of the abscesses at T0, T1 and T© have been presented as LSM ± SE mm³

Variables	Groups										P-values		
	H2O2-gly		O3-oil		PC1		PC2		NC		Group (G)	Time (T)	G × T
	LSM	SE	LSM	SE	LSM	SE	LSM	SE	LSM	SE			
Abscess volume	3.3	0.1	1.9	0.24	5.2	0.47	3.6	0.45	3.3	0.3	< 0.0001	0.25	< 0.0001

Table 6.
Number of ruptured abscesses in experimental groups

	NC	PC 1	PC 2	O ₃ -oil	H ₂ O ₂ -gly
Ruptured Abscesses	0 (None)	14 (73.68%)	11 (64.71%)	2 (2.70%)	2 (2.86%)

decreases in abscess volume over time were apparent for both treatments but not for any control groups. The volume of the abscesses in control groups remained unchanged, if not larger. The volumes of abscesses in the NC and PC2 groups remained unchanged, while in PC1 got larger significantly (*p* = 0.031, Tables 4 and 5). Abscesses in O₃-oil showed significant volume reductions in the first two weeks after treatment. However, a significant reducing effect was only observed after four weeks for H₂O₂-gly. The different responses in treatment vs. control groups showed the therapeutic effects of O₃-oil and H₂O₂-gly on the CLA abscesses.

We observed that O₃-oil caused a significant decline in the abscess volume compared to H₂O₂-gly at T1 (*p* = 0.0002) and T2 (*p* = 0.0288). The reducing effect of O3-oil was significantly higher in T1, compared to H₂O₂ (*p* = 0.0002) and control groups (PC1 = 0.0001, PC2 = 0.0012, NC = 0.0069). Moreover, the volume of the abscesses was greater in PC1 than in NC (*p* = 0.0206). The instillation of olive oil alone increased the volume of the abscess at T1 (*p* = 0.03) and T2 (*p* = 0.01) compared to T0 in PC1. The volume of abscesses showed no significant differences in T1 and T2 compared to T0 in the NC (*p* = 0.366) and PC2 (*p* = 0.211) groups (Tables 4 and 5). It should be noted that the unequal sizes of experimental groups were due to the refusal of the owners to allocate additional animals to the control groups because of observing adverse effects causing obligatory premature termination of the experiment in the control groups. Moreover, the significantly larger abscesses in the H₂O₂-gly group should be accounted as a shortcoming of the study design due to the strict adherence to the rule of Latin square in allocating animals to the groups, while ignoring the importance of the similarity of abscesses in terms of size at the starting point. The number of ruptured abscesses in each group is presented in Table 6. On weekly follow-ups, the owners reported no relapse of abscesses and the complete disappearance of abscesses treated by O₃-oil or H₂O₂-gly.

The effects of gender, species, breed, age, and sex were evaluated as fixed effects. No significant effects were detected (*p* = 0.1457).

Discussion

The most important finding of this study is that the intra-abscess infusion of H₂O₂ and O₃ resulted in a significant decrease in the volume of the abscesses caused by CLA within four weeks (Table 2) to the extent that a considerable shrinkage and/or complete recession occurred on the sixth week.

The surgically opened abscesses should be routinely flushed with disinfectants, such as 10% povidone-iodine or iodine tincture; however, the histotoxicity characteristics of iodine solutions disrupt the healing process of the wounds [34, 35]. Biogenic silver or gold nanoparticles have been used as a postsurgical treatment instead of iodine solutions in cases of CLA with promising results [10, 12]. Moreover, postsurgical administration of antimicrobials could have great value as a part of the preventive strategy against the environmental re-infection of wounds.

The proposed therapeutic procedures apart from surgical intervention are somewhat problematic and inconclusive. The pathophysiology of the disease includes the formation of a thick pyogranulomatous lesion following organism establishment, and the development of an “onion ring” appearance in a process of repeated necrosis of the lesion following pathogenic agent proliferation, which causes the reformation and progressive enlargement of the capsule. This pathologic encapsulation structure precludes the penetration of antibiotics to the pyogenic membrane of the abscess, where the causative agent(s) reside(s) [1].

Combinations of oxytetracycline plus rifamycin or erythromycin have been used for treating CLA [24]. These combinations require the extra-label usage of antimicrobials for long periods, and some of them (e.g., erythromycin) are not labeled for use in food animals. Therefore, these shortcomings restrict their effectiveness either for food animals or other specific species [36]. A combination of subcutaneous usage of procaine penicillin G after opening, draining, and flushing the abscesses and/or intralesional administration of tulathromycin following a closed system lavage has been reported. The procedure has the same problems of requiring extended withholding times and extra-label usage of antimicrobials [23]. Collectively, the usage of antibiotics in the treatment of CLA has not been advocated extensively by veterinarians.

An alternative procedure similar to the present

study is the intra-abscess instillation of formalin, which is advised by some veterinarians [36]. Formalin is carcinogenic and not approved by the FDA. Moreover, it requires waiting for the maturation of the abscess, when it gets softness determined by palpation [36]. The latter point is a restriction to mass medication of the affected animals. Although not approved by the FDA yet, O_3 -oil and H_2O_2 -gly can be instilled into the immature abscesses, which makes it possible to treat all the affected animals in the flock in a single operational setting. This procedure lacks either the adverse effects of iodine solutions or the need for the extra-label usage of antimicrobials.

The mechanisms of action of H_2O_2 (as a 3% solution) have been attributed to directly killing microorganisms, and indirectly reacting as a signaling molecule or second messenger that stimulates effector cells to respond [18]. It has been reported that mild oxidative stress imposed on biological components by O_3 has several beneficial outcomes when used intralesionally in humans [37], providing fast cleansing of wound surface from pyonecrotic masses, stimulating the formation of granulation tissue, and enhancing wound healing [38]. Ozone reacts with the double bonds of triglycerides in vegetable oils, resulting in the production of ozonides and peroxidic species, which exert antimicrobial activity [39]. It is deemed that incorporating O_3 or H_2O_2 in olive oil or glycerin, respectively, extends the effects of these agents that react instantly per se. Antibiotics are deactivated in an abscess fluid environment due to low pH and oxygen tension, high protein content, high bacterial count, deactivating enzymes, and sequestration of bacteria engulfed by leukocytes [40, 41]. The agents O_3 -oil and H_2O_2 -gly retain their activity in purulent environments.

Several vaccines have been marketed against CLA, with varying results [36, 42, 43]. It should be noted that commercial vaccines mainly activate humoral responses and have some points of strength. First, they can limit the spread of an infection rather than eliminate an established infection. In other words, they are not suitable for the cure of affected animals, but their primary benefit lies in preventing the colonization of infection in vaccinated animals if used before their exposure to the organism [36]. Second, their potential for reducing the incidence of external and internal CLA lesions has been shown experimentally [4, 44-47]. Other interventions, including O_3 -oil and H_2O_2 -gly, lack such an important advantage.

In the present study, CP, as the leading causative agent of CLA, was isolated from 87 (48.3%) subjects, followed by a variety of other microorganisms (Table 2). In three abattoir studies, CP was isolated from 12.6% of cases in Iran [48], 34.7% in Poland [49], and

43.7% in Brazil [50]. Other researchers reported that CP was isolated from 27.84% of abscesses from clinically ill sheep and goats and condemned internal abscesses during meat inspection in Saudi Arabia [51]. In Egypt, CP was isolated from 90.07% of clinically infected cases [52]. In contrast, the major isolated bacteria from CLA cases in Spain was *Staphylococcus aureus* subspecies *anerobius* (44.4%), followed by CP (26.3%) [53]. Almost invariably, various other microorganisms were isolated in different studies, which could partly explain the variable results of vaccination against CLA in different geographical locations.

Mandibular lymph nodes were primarily affected in the present study followed by parotids, retropharyngeals, prescapulars, supramammaries, prefemorals, and right popliteals (Table 3). These data suggest that the lymph nodes of the head area are mostly affected, which might be due to the cumulative effects of the feeding behavior with the harsh environment as well as low-quality available range roughages in the desert (e.g., *Tamarix* and *Haloxylon* shrubs). This finding is consistent with the findings of other studies (33, 54, 55). The parotid lymph nodes were affected with higher frequencies in goats in Ethiopia and Egypt [52, 56, 57], while in Spain retropharyngeals were the most affected lymph nodes [58]. It seems that the lymph nodes of the head region are most affected by CLA.

Conclusion

The results of the present study show that the instillation of O_3 -oil or H_2O_2 -gly provides a promising result for the treatment of CLA abscesses in an early period of abscess growth. The authors believe that such a therapeutic intervention is highly needed due to the fact that solely relying on the management strategies have been failing in preventing the spread of the disease [59], so far. The managerial considerations are essential in preventing the discharge of the purulent contents of the abscess into the environment. The formation and rupture of superficial abscesses result in the release of large numbers of bacteria into the environment [1], which plays a vital role in the spread of CLA [60]. The microorganism(s) is (are) able to survive in the environment for several weeks [4]. In the present study, the intra-abscess instillation of O_3 -oil and H_2O_2 -gly prevented the spread of purulent contents into the environment in addition to decreasing the size of the abscesses, especially when they are immature. Moreover, in contrast to antimicrobials, no withdrawal time should be considered for O_3 -oil and H_2O_2 -gly. Additionally, contrary to antimicrobials that would be deactivated in purulent secretions, O_3 -oil and H_2O_2 -gly retain their

activity in an abscess environment, lacking concerns regarding antimicrobial residues for public health.

Future research is warranted, for example, to combine the instillation of O_3 -oil and/or H_2O_2 -gly into the abscesses and vaccination strategy when the internal abscess is also a matter of concern and de-

Materials and Methods

Study approval and Ethical Considerations

Ethical approval of the current experiment was issued by the Committee for Animal Welfare at the Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, IRAN, (approval 3/48948 session 556-28/11/1397).

Methodology

1. Study Design, Inclusion Criteria and Data Collection

This prospective clinical case-control study was conducted in the field in the lowlands of Zirkuh district with the centrality of Hajjiabad, located in south Khorasan province, East of Iran, adjacent to the Afghanistan border, with a cold and semi-arid mountainous climate and an average annual rainfall of 150 mm. The reference coordinates of the study location based on the GCS system are: 33°36'18"N, 59°59'36"E. All the animals in this region, which were referred to the authors with CLA during September 2018 August 2019 were included in this study. Animals with suppurative superficial lymphadenitis were considered eligible for allocation to the study only if they did not have: 1) any apparent clinical sign(s) consistent with a systemic infection, or 2) a history of receiving any local and/or systemic antimicrobial therapy for at least two weeks before the start of the study. Moreover, animals that treated with antimicrobials during the study or those that were not available for examination during the entire study period were retrospectively excluded from the dataset. The population in the present study mainly consisted of a native goat breed and different sheep breeds, including Balochi, Kurdi, Afshari, Chinese and other crossbreeds. The flocks were kept on traditional nomadic management, grazing mainly sparse ranges and receiving occasionally supplemented feedstuffs, including wheat straw and barley grain. Vitamin/trace mineral supplements were provided for the flocks by the owners.

In the present study, two stable slow-releasing combinations, including an emulsion containing ozonated olive oil and a combination of H_2O_2 with glycerin, were selected to be injected into the intact superficial CLA-abscesses in volumes proportional to the size of each abscess. Animals included in the study were assigned to one of five treatments as follows: 1) no therapeutic intervention (NC, N.28 abscesses, 28 animals), 2) injection of olive oil into the abscess (carrier substance of O_3 , PC1, N.19 abscesses, 17 animals), 3) injection of glycerol into the abscess (carrier substance of H_2O_2 , PC2, N.17 abscesses, 13 animals), 4) injection of ozonated olive oil (O_3 -oil, N. 74 abscesses, 61 animals), and 5) injection of H_2O_2 -glycerol into the abscess (H_2O_2 -gly, N.70 abscesses, 59 animals) (Table 1). A total of 208 lymph nodes were examined from 180 affected sheep and goats in pre-existing groups (some animals had more than one abscess), which were clinically evaluated in terms of general health status and vital signs. The animal characteristics such as species, age, sex, breed, and BCS were also recorded at this stage.

2. Group Assignment

The animals were randomly assigned to each group, irrespective of the anatomical locations, gender of the animals or the size of the affected lymph nodes. Allocation was completed according

to a Latin square, one by one to different experimental groups in five affected flocks on different occasions. The primary agreement between the researchers and owners were to allocate animals to experimental groups equally. However, the owners refused to allocate more animals to control groups, when they observed the poor response of injecting olive oil and glycerol alone into the abscesses, leading to a comparably fewer allocations to these groups.

3. Preparation of the therapeutic agents

Glycerol (1,2,3-propanetriol) (pharmaceutical grade, Merck Co.) was considered the best choice for dissolving H_2O_2 (pharmaceutical grade, Merck Co.) in terms of viscosity, availability and price, which could effectively maintain intra-abscess H_2O_2 concentrations while avoiding irritating effect of a pure 3% w/v H_2O_2 solution.

For each patient assigned to H_2O_2 -gly, approximately 1 ml of 3% w/v H_2O_2 solution (147 mmol/L) was thoroughly mixed with 5 ml of 1% w/v glycerol (1:5 ratio) immediately before injection under a treatment protocol introduced by Japanese investigators (KORTUC II protocol) [17, 19, 22] to obtain a final 0.5% H_2O_2 in glycerol solution.

Olive oil was chosen as a solvent for a mixture of oxygen and O_3 gases, resulting in the ozonated olive oil. The product has a longer half-life, and is more stable and applicable in field settings than the original gaseous O_3 . Ozonated oils, are considered well-tolerated and safe compounds (LD50 > 2000 mg/kg BW), and remain stable at room temperature for up to one year and in the refrigerator for up to two years, provided to be stored in dark glass bottles to avoid sunlight. These properties altogether have led to the commercialization of pharmaceutical products containing ozonated oils for a variety of purposes [17, 26-28]. Sterile injectable emulsion of ozonated olive oil was prepared from sterile raw materials and packed in a class B environment in 10 ml amber vials aseptically. The emulsion was prepared by mixing 0.35% W/V of ozonated olive oil with sterile water for injection and 2% W/V of Tween 80[®] as an emulsifier in a laboratory scale homogenator. The peroxide, acid and iodine index of ozonated olive oil were 2439 ± 13.3, 17.3 ± 0.06, and 0, respectively [United States Pharmacopeia and the National Formulary (USP 35 - NF 30), Rockville (MD): The United States Pharmacopeia Convention; 2012].

Formulation and preparation of an injectable sterile solution of H_2O_2 3% w/v and glycerol 1% w/v (1:5 ratio), as well as an injectable sterile emulsion containing ozone in olive oil were performed in the school of Pharmacy, Mashhad University of Medical Sciences (MUMS).

No side effects, including the signs of pain or restlessness were manifested by the animals following the instillation of H_2O_2 -gly or O_3 -oil.

4. Clinical Examination and Sample Collection

Rectal temperature, as well as respiratory and heart rates for each animal were measured, and the conjunctival, oral and vulvar (in females) mucous membranes were examined to assess the general health condition of the animals. In addition, BCS was judged on a 1-5 numerical scale [28]. Superficial abscesses were identified and the location was recorded for each individual. The three dimensions (length, width, and height) of each abscess were measured in millimeters using a manual analogue Vernier calliper (WOLFOX[®], 127 mm), after thorough washing and shaving. The abscess volume (almost ellipsoid in shape in mm³) was extrapolated from the dimensions using the following equation: Abscess Volume = $\pi/6 \times L \times W \times H$ [20].

The abscesses measurements were conducted at the time of inclusion in the study before treatment (baseline, T0) and again after two and four weeks. A fourth examination was conducted in the sixth week after treatment; however, the majority of abscesses in the control groups had been ruptured, while most of the abscesses were recessed in the treatment groups. The statistical

analysis was limited to T0, T1, and T2 with intervals of 2 weeks. Further follow-ups were carried out by calling the owners weekly.

5. Experimental procedures

The exposed surface of the abscesses was thoroughly washed and aseptically prepared with povidone-iodine surgical scrub, followed by wiping with a 70% isopropyl alcohol, letting to dry. The abscesses were punctured using disposable 18 G × 1.5" needles armed with a 2.5 ml syringes. The abscesses contents were aspirated, while the tip of the needle was located at the opposite side of the puncture site, presumably near the pyogenic membrane of the abscesses. Aspirated materials were immediately transferred to a sterile, tightly capped tube. Samples were sent forwarded to the laboratory following strict recommendations regarding the shipment of microbiological specimens [29].

Prepared experimental agents corresponding to the respective experimental groups were then injected through the needle still in place. The animals assigned to NC did not receive any instillation, while abscesses in PC1 and PC2 were instilled with olive oil and glycerol without active compounds, respectively. The animals assigned to the treatment groups (O₃-oil and H₂O₂-gly) received either ozonated olive oil or an emulsion containing H₂O₂ in glycerol into the abscesses, respectively. The instillation was continued until the abscesses were felt to be filled by palpation. Therefore, variable amounts of solutions were instilled into the abscesses, which were recorded for all of them.

6. Bacterial Isolation and Identification

Samples were streaked onto Columbia agar supplemented with 8% defibrinated sheep blood and MacConkey agar. Blood agar plates were subsequently incubated in pairs for 48 h at 37°C, one aerobically and the other microaerophilically (candle jar). The pure isolates were identified based on colony characteristics, hemolysis pattern, microscopic cell morphology, and biochemical tests, namely catalase, oxidase, O/F test, SIM, gelatin hydrolysis, urea hydrolysis, nitrate reduction, MR/VP test, CAMP or reverse CAMP test, and carbohydrate fermentation profiles [30-33].

7. Data analysis

The current study was conceived with four evaluation time points T0, T1, T2, and T3, where T0 was the baseline immediately prior to the administration of experimental treatments and each following time point was two weeks after the previous one. Statistical analysis was limited from T0 to T2, as too few values for T3 were available for a meaningful analysis.

A difficulty for analysis was that the data of ruptured abscesses were missed after rupture. Assuming that larger abscesses were more likely to rupture than smaller abscesses, a missing value at one of the time points due to a ruptur decreases the mean abscess volume of the corresponding treatment and it thereby results in a bias that becomes increasingly important when more abscesses rupture in one treatment. In an attempt to quantify this effect, each abscess was assigned a categorical value for the parameter "treatment failure". The value 0 was assigned to the each abscesses with a volume of 80% or smaller than that of T0 at T1 or T2 that was 80% or less of the volume determined at T0. The value 1 was assigned to an abscesses with a volume of at least 80% of T0 at T1 and T2, and the value 3 was assigned to theabscesses that ruptured between T0 and T2. The cut-off value was set arbitrarily. Too few animals with data in T3 were available to be included in the statistical analysis.

8. Statistical analysis

Unless stated, the results are expressed as LSM ± SEM or as a median and interquartile range for variables not meeting the assumption of normality. The statistical significance level was set at *p* < 0.05. Data were tested for normal distribution and homo-

geneity of variance using Proc UNIVARIATE, and abscess volumes were square root transformed to achieve normal distribution.

To analyze of the square root transformed key outcome variable "abscess volume" a repeated measures analysis of variance was conducted using PROC MIXED. The animal ID was considered subject; as subject to determine the fixed effects of treatment, time, as well as the interaction between treatment and time, with time as repeated factor on the abscess volume. Covariates included in the initial model were sex, breed, and species of study animals. The covariates that were not statistically significant were removed from the initial analysis to obtain the definitive model. The autoregressive1 covariance structure was chosen based on the lowest Akaike information criterion. Bonferroni-adjusted *p*-values were used to assess differences between treatments at specific sampling times and also differences between sampling times whenever the F test was statistically significant. Proc FREQ applied frequency analyses on categorical variables, such as "treatment failure" and "pathogen identified in the abscess".

For convenience and because no preliminary data were available to make reasonable assumptions on treatment outcomes, the sample size of the present study was not based on a power analysis but on a convenience sample size that was all animals eligible for inclusion presented during one year.

All analyses were conducted with SAS software (SAS 9.4, SAS Inst. Inc. Cary, NC).

Authors' Contributions

G.A.K: examination of the animals, sampling and writing the manuscript. R.M.: Performing microbiological culture and examination procedures. O.M.: Preparation and quality control of the injectable products. B.N.: Finding the eligible flocks, examination of the animals, sampling, extensive participation in the process of writing the manuscript, and leadership of the field operations. M.A.B.: Supervision and planning the bacterial culture and examination procedures. K.S.: Designing the study, planning the field operations, writing the manuscript. All the authors have critically reviewed the manuscript.

Acknowledgements

The authors wish to thank Walter Gruenberg, Faculty of Veterinary Medicine, Giessen, Germany, for performing the statistical analysis, as well as suggesting nice comments and hints. Moreover, we would like to thank the Dean for Research, Ferdowsi University of Mashhad, for financial and the technical support of the project (approval, no. 3/48948 session 556-28/11/1397). The field part of this study was accomplished under the leadership of the 4th author, Drs. B. Norouzi, who made impossible, possible.

Competing Interests

The authors have nothing to disclose.

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How to cite this article

Khorshidian Gh.A, Moradi R, Rajabi O, Norouzi B, Askari Badouei M, Sharifi K. Therapeutic Intervention for Caseous Lymphadenitis Using Intra-abscess Instillation of Ozone or Hydrogen Peroxide in Small Ruminants. *Iran J Vet Sci Technol*. 2023; 15(3): 16-26. DOI: <https://doi.org/10.22067/ijvst.2023.82086.1246> URL:https://ijvst.um.ac.ir/article_44373.html